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Abstract: Liver is an attractive organ for gene delivery in order to correct various genetic (metabolic) diseases. Hydrodynamic vein injection of naked DNA/minicircles devoid of viral or plasmid backbones was demonstrated in, for example, murine phenylketonuria to allow sustained therapeutic transduction of hepatocytes. Here we show successful hepatocyte transfusion in domestic small pigs immediately after weaning upon portal vein catheterization and hydrodynamic injection of naked DNA/minicircle vectors expressing the luciferase gene from the CMV or a liver-specific promoter. First, we established a surgical method allowing hydrodynamic portal vein pressurization up to 120 mmHg and infusion of naked DNA in pigs (n = 5) with long-term survival. No acute adverse effects such as changes in liver transaminases or signs of liver cell damage were observed. We then showed efficiency of stable hepatocyte transfection at 10 and 28 days in single experiments (n = 7) where we found that up to 60% of samples (45/75) were polymerase chain reaction (PCR)-positive for minicircle-DNA. Of these samples, 13% of the positive specimen (6/45) showed low but stable luciferase expression when driven by a liver-specific promoter, as well as appropriate copy numbers per diploid genome. In conclusion, we accomplished a safe procedure for stable transfection of liver cells upon hydrodynamic gene delivery using minicircle vectors in small pigs as a prerequisite to potentially treat infants with genetic liver diseases.

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Hepatocyte Transfection in Small Pigs After Weaning by Hydrodynamic Intraportal Injection of Naked DNA/Minicircle Vectors

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Liver is an attractive organ for gene delivery in order to correct various genetic (metabolic) diseases. Hydrodynamic vein injection of naked DNA/minicircles devoid of viral or plasmid backbones was demonstrated in, for example, murine phenylketonuria to allow sustained therapeutic transduction of hepatocytes. Here we show successful hepatocyte transfusion in domestic small pigs immediately after weaning upon portal vein catheterization and hydrodynamic injection of naked DNA/minicircle vectors expressing the luciferase gene from the CMV or a liver-specific promoter. First, we established a surgical method allowing hydrodynamic portal vein pressurization up to 120 mmHg and infusion of naked DNA in pigs ($n=5$) with long-term survival. No acute adverse effects such as changes in liver transaminases or signs of liver cell damage were observed. We then showed efficiency of stable hepatocyte transfection at 10 and 28 days in single experiments ($n=7$) where we found that up to 60% of samples (45/75) were polymerase chain reaction (PCR)-positive for minicircle-DNA. Of these samples, 13% of the positive specimen (6/45) showed low but stable luciferase expression when driven by a liver-specific promoter, as well as appropriate copy numbers per diploid genome. In conclusion, we accomplished a safe procedure for stable transfection of liver cells upon hydrodynamic gene delivery using minicircle vectors in small pigs as a prerequisite to potentially treat infants with genetic liver diseases.

INTRODUCTION

NORMAL LIVER FUNCTION IS ESSENTIAL for the mammalian organism in multitudinous aspects. The hepatic integrity can be threatened by a large variety of acquired and inherited disorders; in the former category, trauma, infections, cancer, autoimmune disease, and many other disorders can lead to hepatopathy. In addition, inherited diseases can lead to impairment of any of the numerous biochemical pathways located in the liver, and some of these pathways are with respect to pathophysiology even limited to this organ.[1] An example for the latter is the main pathway for ammonia detoxification, the urea cycle, which is composed of several consecutive enzymatic steps.[2–4] Importantly, failure in just one step of this hepatic pathway leads primarily to accumulation of neurotoxic metabolites and brain dysfunction[5–8] although the defect is expressed only in the liver. The presence of such disorders, with defects only in the liver but severe sequelae for the entire body, makes the liver a primary target organ for novel (not only) gene therapeutic approaches.[9,10]

Currently available treatment modalities are, at least in the case of metabolic liver disorders, far from being satisfactory since they often address only the symptoms of disease. In addition, several curative approaches were evaluated such as transplantation

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of parts of the whole liver or of hepatocytes or of hepatocyte-like cells derived from stem cells.[11–13] While liver transplantation has proven beneficial for a growing list of disorders, hepatocyte or stem cell infusions may rather have a bridging than a curative potential. As an alternative procedure, gene therapy with different vector strategies was developed. Most trials were undertaken with viral vectors that allowed effective transfection of liver cells but raised several concerns, including safety of repeated applications or of the risk for mutagenesis. As a likely less immunogenic approach, nonviral vectors were recently introduced to the field of gene therapy.[14,15]

Nonviral vectors are circular naked DNA molecules devoid of any viral or plasmid components and require other routes of administration than viral vectors in order to achieve sufficient rates of transfection. Several studies in small rodents (mice) have shown that hydrodynamic injections in the tail vein allow for effective transduction of liver cells (see for instance ref.[16]). If the liver is to be addressed in larger animals or humans, hydrodynamic injections may require direct vascular access via the portal or hepatic veins. Respective attempts to prepare such interventions for use in human patients have already been made in single large animal studies where portal vein injections were auspiciously applied in adult pigs or more recently also in dogs.[17–20] Previous interventions in human newborns receiving hepatocyte transfusions were done via access to the portal vein by surgical insertion of a Hickman catheter into the middle colic vein or by noninvasive interventional catheter placement in the left portal vein branch.[21] However, a direct surgical access by catheter placement into the portal vein is a challenging procedure in newborns and not at all yet part of clinical routine.

In this study, we test the hypothesis that our successful hydrodynamic injection protocol of minicircles (MCs) in adult mice,[16] done via the tail vein, can be adapted to portal vein injections in small domestic pigs immediately after weaning. We regard this as a prerequisite before naked DNA can be included in the armamentarium of gene therapeutical approaches aiming for cure of genetically determined liver diseases in newborns or infants with inherited (metabolic) disorders that are located in the periportal area of the liver (as it is the case in urea cycle defects). The purpose of this study was therefore to develop a safe surgical method to perform direct hydrodynamic portal vein injections of MCs in small pigs, to achieve long-term survival of the animals, and to evaluate the efficacy for clinical application of such an experimental approach.

MATERIALS, METHODS, AND ANIMAL HANDLING

Naked DNA/MC vectors

Purified MC vector MC.CMV-luc (3882 bp) was purchased from PlasmidFactory (Bielefeld, Germany) harboring the firefly luciferase gene expressed from the (nonliver specific) CMV promoter. A derivative MC vector MC.P3-luc (2324 bp) expressing the firefly luciferase from the liver-specific promoter P3[16,22] was generated and purified according to a previously published method.[16,23] More detailed information on this vector can be obtained upon request.

Animal handling

Animal experiments were performed in accordance with the guidelines and policies of the Veterinary Office of the State of Zurich and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Animal studies received approval from the Cantonal Veterinary Office, Zurich, and the Cantonal Committee for Animal Experiments, Zurich, Switzerland (permission for animal experiments Kt ZH 34-2013). Four-week-old female domestic pigs (weight 3.6–7.5 kg; Table 1) were separated from their mothers immediately after weaning and brought to a loose barn with porcine mates 7 days before surgery.

Surgical procedures

A median laparotomy was performed, followed by isolation of the different structures of the hepatic ligament. The portal vein and hepatic arteries were separated from the common bile duct. Then, a circular suture (Prolene 7-0) was placed and a portal venous cut down was performed in order to place a 7 Fr two-lumen catheter directly into the main portal vein. The position of the catheter was ensured and corrected by radiography to place the tip of the catheter into the main portal vein and thus secure injection of MCs into the entire liver.

Table 1. *Characteristics of pigs used for intraportal injections of MC-DNA vectors*

Pig no.[a]	*Body weight on surgery day (kg)*	*Time of sacrifice and sample procurement (days)*	*Body weight (kg) on day of sacrifice*	*Liver weight (g) on day of sacrifice*
A1	4.3	28	11.0 (day 28)	241
A2	4.8	28	10.0 (day 28)	254
B1	7.5	28	15.5 (day 28)	388
B2	7.0	28	15.0 (day 28)	406
C	5.6	10	8.8 (day 10)	246
D1	3.6	10	4.8 (day 10)	135
D2	7.0	10	n.d. (day 10)	400

n.d., not determined; MC, minicircles.
[a]Female pigs.

The catheter was tightly fixed by the circular suture. For all experiments, clamps were placed at the hepatic arteries and the portal vein distal from the catheter allowing hepatic inflow obstruction (Fig. 1A, C–F). Likewise, the suprahepatic inferior cava vein was clamped in order to prevent venous outflow from the liver during the injections according to previous reports with adult pigs (Fig. 1A, C–F).[17,18] The total clamping time was kept short (≤10 min) given the known vulnerability of the pig intestine to outflow obstruction.

Establishment of intraportal injection of small pigs

The surgical and injection procedures for portal vein infusion were set up with five small female pigs in acute studies; that is, animals were sacrificed maximally 6 hr after completion of the procedure while still under deep anesthesia. Anesthesia was introduced with propofol and sustained with isoflurane during surgery. Continuous monitoring including central venous pressure measurement via the jugular vein, arterial blood pressure, transcutaneous oxygen saturation, heart and respiratory rates, and temperature was installed and kept throughout the procedure. Intravascular pressure in the main portal vein during hydrodynamic injections was monitored via measurement at the tip of one lumen of the catheter (Fig. 1B). Placement of the catheter in the main portal vein was controlled by angiography. During establishment of hydrodynamic injections, flow rate, time, and volume of the injections were modified with flow rates between 10 and 20 ml/sec and injection time between 1 and 3 sec. The naked DNA/MC vectors were diluted in 30 ml of 0.9% normal saline (Braun Medical AG, Sempach, Switzerland) for adjusting concentrations as indicated. Final conditions were controlled under angiography to document optimized intrahepatic exposure time of the injected solution.

When the procedure was established, we subjected seven small female pigs immediately after weaning to hydrodynamic portal vein infusion with luciferase-expressing MC vectors for chronic or long-term expression studies (Tables 1 and 2). Pigs A1 and A2 were injected with 0.44 mg of MC.CMV-luc, while pigs B1 and B2 were injected with a 10 times higher concentration (4.4 mg), and pig C with 2 mg of the same MC vector. Whereas in pigs A and B the flow rate of the injection was 20 ml/sec, in pig C a slower rate of 10 ml/sec was used. The same conditions were applied in pigs D1 and D2, which were injected with 2 mg of vector MC.P3-luc carrying the synthetic liver-specific promoter P3.[16] It should be noted that the CMV promoter fragment is roughly 1.9 kb, while the P3 promoter has a length of 0.3 kb. In the following, we analyzed the distribution and fate of MC-vector DNA, as well as stable gene expression in the liver of these animals.

Postoperative monitoring

Animals were kept for 4 weeks ($n=4$) or 10 days ($n=3$) after surgery. Liver transaminases (aspartate aminotransferase, AST; alanine aminotransferase, ALT), lactate dehydrogenase (LDH), and C-reactive protein (CRP) were measured in the clinical chemistry laboratory at University Children's Hospital Zurich, Zurich, Switzerland, by automated analyzer UniCel DXC600 (Beckman Coulter, Nyon, Switzerland) every week, except for pigs A, in which measurements started on day 15 after surgery because permission for collecting blood and urine was not yet available. Baseline levels were obtained before surgery (CRP was not measured at that time).

Collection of samples

Samples of blood and urine for monitoring of standard biochemistry and for MC determination were taken once a week under general anesthesia. Pigs were sacrificed under general anesthesia with propofol 10 days ($n=3$) or 4 weeks ($n=4$) after MC injection. The entire liver was removed before collection of 75 samples from all 5 lobes. Figure 2 shows the distribution of the 75 samples that were collected and shock-frozen immediately after resection. In addition, samples from heart, spleen, diaphragm, lung, and kidneys were collected and stored shock-frozen. For histology of the liver, tissue was fixed in formalin (4%). Following procurement, liver samples were also stained for hematoxylin–eosin (H&E) in order to analyze the amount of necrosis at time of euthanasia.

Analysis of MC vector-DNA in pig liver

Liver tissue was homogenized using the TissueLyser II (Qiagen, Hombrechtikon, Switzerland). DNA was extracted from liver homogenates using the "DNeasy Blood and Tissue Kit" (Qiagen). This kit was used as indicated in the manufacturer's protocol except for all steps before loading on the membrane, where the amount of all fluids was doubled. Purity and quality of DNA were measured with NanoDrop ND1000 (Thermo Fisher Scientific). Polymerase chain reaction (PCR) with DNA isolated from the tissue samples was performed to determine presence of MC. As forward primer 5′-CAC GTT CGT CAC ATC TCA TCT ACC-3′ (primer f2 luc) and as reverse primer 5′-TGA GCC CAT ATC CTT GTC GTA TCC-3′ (primer r2 luc) were used,

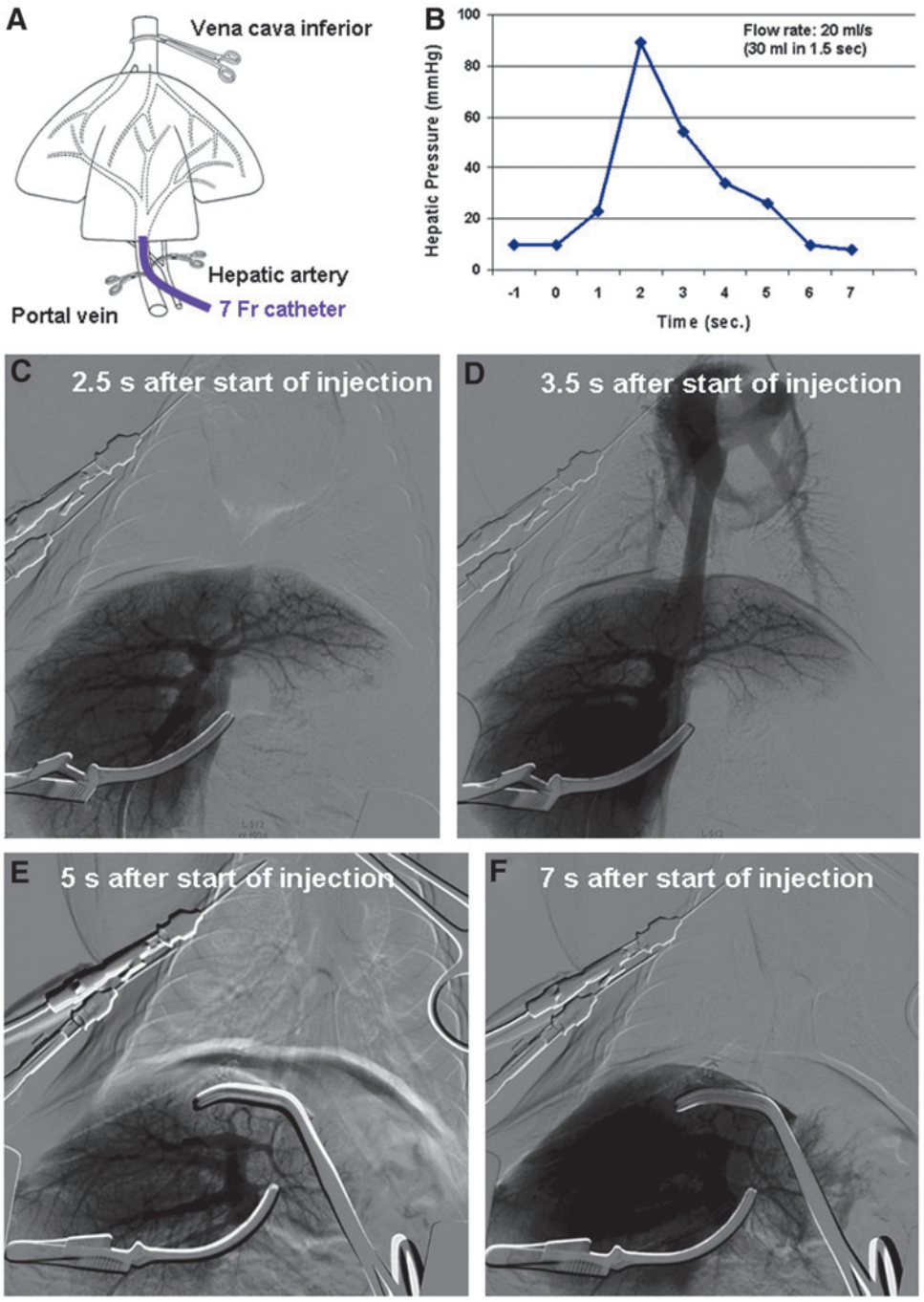

Figure 1. Scheme of the portal vein access, pressure curve, and angiography. **(A)** Scheme of a liver with portal vein, hepatic arteries, and hepatic veins, the latter leading to the inferior vena cava. For the hydrodynamic injection, the catheter was placed directly in the main trunk of the portal vein. During the injection the portal vein, hepatic arteries and the suprahepatic inferior vena cava were clamped to allow pressure generation. **(B)** Pressure curve for pig A2 as a representing example was plotted during hydrodynamic injection with a sensor at the tip of the catheter, confirming the fast and significant increase of the portal vein pressure. **(C–F)** Contrast medium was injected by hydrodynamic injection in the same way as later for the chronic experiments. Distal portal vein and hepatic arteries were clamped in all experiments for the duration of the injection. Pictures were taken from a film with 6 images/sec. **(C)** and **(D)** represent the situation from an experiment without clamping of the suprahepatic vena cava, while there was clamping of the latter vein in the experiment shown in **(E)** and **(F)**. **(C)** shows the distribution of contrast medium 2.5 sec after the injection limited to the liver. **(D)** Picture taken 3.5 sec after the injection shows that a substantial portion of the contrast medium has already escaped to the right heart and pulmonary arteries. **(E)** and **(F)**, obtained 5 and 7 sec after the injection, demonstrate that clamping of the suprahepatic vena cava was efficient in preventing early loss of the injected solution to the circulation. Color images available online at www.liebertpub.com/hgtb

Table 2. *Portal pressure and flow rate during intraportal MC-DNA injection, MC-vector type and amount, and number of positive PCR samples*

Pig no.	Maximum portal pressure achieved during MC injection[a] (mmHg)	Flow rate (ml/sec); injection time (sec)	Injected MC[b] (mg)	Number of PCR-positive liver samples
A1	89	20; 1.5	0.44 (MC.CMV-luc)	8/75 (11%)
A2	93	20; 1.5	0.44 (MC.CMV-luc)	n.a.
B1	51	20; 1.5	4.4 (MC.CMV-luc)	19/75 (25%)
B2	n.d.	20; 1.5	4.4 (MC.CMV-luc)	n.a.
C	21	10; 3	2.0 (MC.CMV-luc)	9/75 (12%)
D1	45	10; 3	2.0 (MC.P3-luc)	45/75 (60%)
D2	n.d.	10; 3	2.0 (MC.P3-luc)	36/75 (48%)

n.a., not analyzed; n.d., not determined because of technical problems during injection; polymerase chain reaction, PCR.

[a]Baseline 5–12 mmHg.

[b]The size of MC vector MC.CMV-luc is 3882 bp, while that of MC.P3-luc is 2324 bp.

both of which binding specifically to the luciferase gene on the MC.CMV-luc, while primer f2 luc and reverse primer 5′-TAG GCC CAT ATC CTT GCC TGA TAC (primer r3 luc) were obtained to amplify MC.P3-luc. PCR for 42 cycles using HOT FIREPol polymerase (Solis BioDyne, Luzerne, Switzerland) was performed at an annealing temperature of 58°C. The amplified fragment had an expected length of 533 bp. As positive control we used DNA isolated from wild-type mouse liver after hydrodynamic tail vein injection[16] with the MC.CMV-luc, which was positive upon IVIS analysis (*in vivo* imaging system; H.M. Viecelli and B. Thöny, unpublished). PCR products for both MC vectors (533 bp) were control sequenced using the BigDye Terminator cycle sequencing kit version 1.1 on a 3130/3130xl Genetic

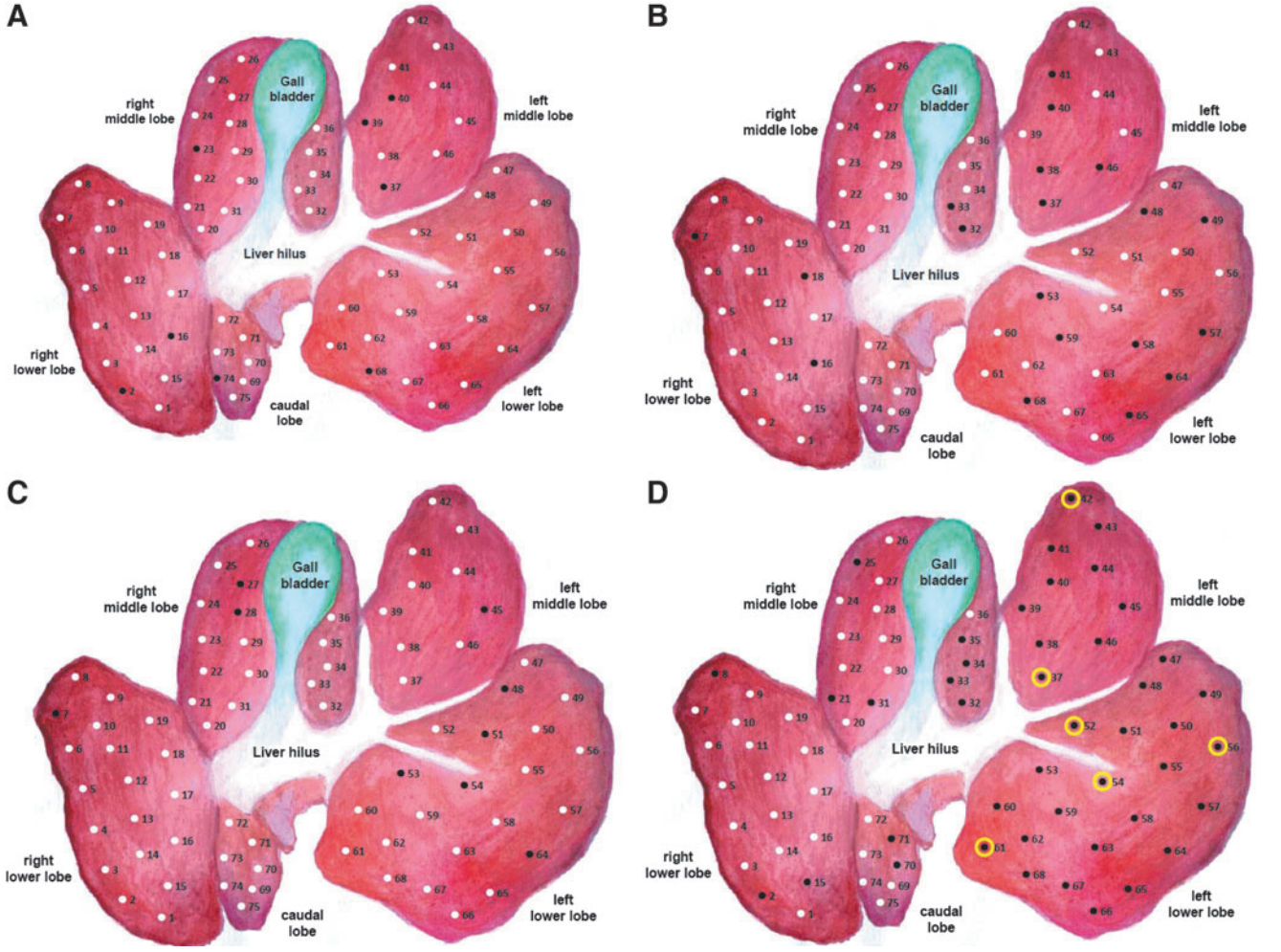

Figure 2. Anatomical sketches of pig liver lobes and efficacy of stable naked DNA/MC delivery and luciferase expression upon intraportal infusion. Drawing of a caudal view on pig liver with position of the collected tissue samples indicated by white dots (if PCR-negative) or black dots (if PCR-positive); PCR-positive samples that are positive also for luciferase activity are indicated by black dots encircled with yellow. **(Pig A1)** Results shown represent the findings in pig A1, which was injected with 0.44 mg of MC.CMV-luc and sacrificed 4 weeks after surgery. **(Pig B1)** Results shown represent the findings in pig B1, which was injected with 4.4 mg of MC.CMV-luc and sacrificed 4 weeks after surgery. **(Pig C)** Results in pig C, which was injected with 2 mg of MC.CMV-luc at slower infusion rate than for pigs A and B (10 ml/sec instead of 20 ml/sec) and sacrificed 10 days after surgery. **(Pig D1)** Results in pig D1, which was injected with 2 mg MC.P3-luc at an infusion rate of 10 ml/sec and sacrificed 10 days after surgery. Color images available online at www.liebertpub.com/hgtb

Analyzer (ABI sequencer; Applied Biosystems, Zug, Switzerland). Forward and reverse primers were used as described for PCR above. The received sequence was matched with the one of luciferase. The same procedure was applied to samples from heart, spleen, diaphragm, lung, and kidneys obtained when animals were sacrificed.

Analysis of MC vector-DNA in pig blood and urine

To investigate the MC latency in the porcine organism and to determine the washout characteristics of MCs, blood and urine were investigated at baseline and weekly after surgery until euthanasia. Urine and plasma samples were directly used as template for PCR without prior DNA isolation. If urinary creatinine was above 7.6 mM, the sample was diluted with water to reach a creatinine value below this threshold, which was determined before by PCR of urine samples with known creatinine levels (samples with a creatinine higher than the threshold inhibited the PCR so that even positive samples yielded a negative PCR result; data not shown). Plasma samples were diluted 1:10 with water for the same reason as described for the urine samples. Threshold was determined by PCR of different dilutions of plasma. PCR amplification was done as described above. As a positive control, MCs in urine, plasma, and water were amplified, and as a negative control, untreated urine and plasma was used. The PCR products with a length of 533 bp were confirmed by sequencing as above.

Copy number assay

Genomic DNA (gDNA) from liver tissue was isolated using DNeasy blood and tissue kit (Qiagen). In accordance with the manufacturer's protocol, 100 ng of gDNA from each sample was used as a template. Standard curves plotting cycle threshold (Ct; y axis) against log vector copy number (x axis) for each vector infused in pig livers were generated using serially diluted DNA vector with various copy numbers (2×10^7 copies to 20 copies) with $y=-3.3x+35.6$, $R^2=0.999$ and $y=-3.3x+37.7$, $R^2=0.998$ for MC.CMV-luc and MC.P3-luc, respectively, along with 100 ng noninfused control gDNA. The number of vector genomes per cell in liver tissue was determined by absolute quantitative PCR analysis using TaqMan® gene expression assay corresponding to luciferase (Mr03987587_mr; Life Technologies, Zug, Switzerland) in all liver tissue samples that were positive in end-point (saturating) PCR. Quantitative PCRs were performed by ABI PRISM 7900 sequence detector, and the data were analyzed with Sequence Detection System (Life Technologies). The haploid genome size of pig is estimated to be 2800 Mb (according to NCBI Genome Database) and the mass of a single diploid copy is 6.14 pg, which was calculated according to the description from Life Technologies. Therefore, the 100 ng gDNA contains 16,287 copies of diploid genome (1×10^5 pg/6.14 pg). The average transgene copy number in 100 ng gDNA in each liver sample was then divided by 16,287 to obtain the vector copy number in each cell. For comparison to rodent liver, we injected a wild-type C57BL/6 mouse with 15 μg of MC.P3-luc via hydrodynamic tail vein injection.[16] The standard curve with a serial dilution of known vector copy numbers (2×10^7 copies to 20 copies) was $=-3.0x+38.8$, $R^2=0.999$.

Luciferase activity

To study expression levels of the naked DNA injected, we measured luciferase activity in all collected 75 liver samples. For this, we used the Luciferase Assay System (Promega, Dübendorf, Switzerland) on the microplate reader infinite F 200 (Tecan, Männedorf, Switzerland). For data analyses, i-control 1.10 software (Tecan) was used. As a positive control, liver tissue lysates from two PKU mice injected with MC expressing luciferase were used (not shown). As a negative control, a liver tissue lysate of an untreated pig was taken.

RESULTS

Establishment of surgical procedures for portal vein injections in small pigs

Based on what has been pioneered by others performing hydrodynamic liver infusion of naked DNA in adult pigs, we reasoned that a flow rate between 10 and 20 ml/sec, an injection volume of 30 ml, and a vascular pressure in the portal vein between 50 and maximally 120 mmHg (baseline 5–12 mmHg) are considered to be optimal for small pigs.[18,24,25] Accordingly, the surgical procedures and the required injection parameters were first tested and established in small animals ($n=5$), and thereafter applied in pigs with equal size for chronic or long-term studies ($n=7$) of naked DNA/MC vector delivery by hydrodynamic portal vein injection (see below). Importantly, all hemodynamic parameters during clamping of the liver remained normal. During the intraportal injection of 30 ml MC solution in 1.5 or 3 sec, the size of the liver increased transiently and went back to normal within a few seconds after opening the clamps, as reported by others.[18,24,25] In general, intravascular pressure in the main portal vein during hydrodynamic

injections was monitored via measurement at the tip of the (two-lumen) catheter. Placement of the catheter in the main portal vein was controlled under angiography to document optimized intrahepatic exposure time of the injected solution (Fig. 1C–F). Angiography allowed in addition to follow the flow of contrast medium under exactly the same injection conditions as later used in MC experiments. Hereby, we saw, as expected, that waiving of hepatic outflow clamping leads to disappearance of contrast medium within few seconds (Fig. 1C, D), while clamping of the suprahepatic vena cava resulted in efficient sealing of hepatic outflow without a need to clamp in addition the infrahepatic vena cava (Fig. 1E, F). Removal of the catheter, recovery after surgery, and clinical monitoring during the postoperative period were uneventful in all pigs. Animals returned to a loose barn with porcine mates within six hours after the end of anesthesia. There were no complications regarding wound healing and no need for medical interventions during postoperative course, and animals were fed their normal diet upon return to their barn.

In conclusion, all small pigs included in this study (total $n=12$), including the animals for the acute study ($n=5$) as well as for chronic experiments ($n=7$; see below), were stable during surgery, and we did not observe any apparent adverse effect.

Detection of MC-DNA in pig liver after 10 days and 4 weeks following portal vein infusion

Once we had established the basic conditions for intraportal injections, we tested only a limited number of small pigs for gene delivery using MC/naked DNA vectors expressing luciferase either from the CMV or the liver-specific promoter P3[16]. While a total of seven pigs were injected with different amounts of MC vectors, liver tissue samples of five pigs were analyzed in depths (A1, B1, C, D1, and D2; see Tables 1–3 for the various characteristics). Here we compared the effect of different amounts of total DNA vector injected (0.44, 2, and 4.4 mg) as well as the two different promoters for luciferase expression, CMV (1.9 kb) and the liver-specific promoter P3 (0.3 kb), the latter used in two experiments (pigs D1 and D2). Pressure monitoring showed that in all pig livers, a substantial increase of the maximum portal pressure during injection was achieved (Table 2). Besides the 75 tissue specimens representing all 5 liver lobes, samples from lung, kidney, diaphragm, spleen, and heart were analyzed. PCR products were validated by DNA sequence analysis that confirmed the presence of the injected luciferase gene (Fig. 3C). Tissue specimens from all other organs were PCR-negative (not shown).

Table 3. *(A) Copy number per diploid genome in liver of pigs infused with MC vectors, and (B) luciferase activity in liver of pig D1 infused with 2 mg of MC.P3-luc*

(A) Vector genomes per diploid liver genome

	MC.CMV-luc			*MC.P3-luc*	
Liver lobe	*Pig A1 (0.44 mg)*	*Pig B1 (4.4 mg)*	*Pig C (2.0 mg)*	*Pig D1 (2.0 mg)*	*Pig D2 (2.0 mg)*
Right middle lobe	n.d.	1.1×10^{-4}	3.6×10^{-5}	1.2×10^{-3}	4.0×10^{-4}
Right lower lobe	6.9×10^{-7}	1.8×10^{-4}	4.2×10^{-4}	3.7×10^{-3}	4.3×10^{-4}
Left middle lobe	8.4×10^{-4}	1.2×10^{-3}	1.9×10^{-5}	1.8×10^{-1}	1.6×10^{-4}
Left lower lobe	n.d.	4.3×10^{-4}	1.4×10^{-3}	2.3×10^{-1}	2.3×10^{-4}
Caudal lobe	1.4×10^{-6}	n.a.	n.a.	1.8×10^{-3}	3.8×10^{-4}

(B) Luciferase expressed from a liver-specific promoter and vector copy number in liver of pig D1

No. of liver sample (according to Fig. 2)	37	42	52	54	56	61
Luciferase activity (RLU/μg protein)	8.42	3.11	1.52	1.52	6.25	1.06
Copy number per diploid genome	1.5×10^{-1}	9.9×10^{-1}	1.3×10^{-1}	4.1×10^{-1}	2.5	3.5×10^{-2}

n.a., not analyzed for genome copy numbers as end-point (saturating) PCR was negative; n.d., not detectable, represents a Ct value detected by quantitative PCR that was higher than 35; RLU, relative light unit.

In liver, the number of positive samples varied depending on the amount of MCs injected and the flow rate or injection time. For instance, 4 weeks after infusion, pig B1 with 4.4 mg of vector DNA resulted in double the amount of PCR-positive liver samples (19/75 = 25%) as compared with pig A1 with 10 times less vector infused (0.44 mg and 8/75 = 11% positive samples). The majority of the 19 positive samples (14/19) in pig B1 were located in the left middle and left lower lobe (pig B1 in Fig. 2), suggesting that the main part of the injected solution went to the respective liver lobes. Interestingly, a prolonged injection time from 1.5 to 3 sec in pig C compared with pig A1, while the injection volume was identical, resulted in 4-fold lower portal pressure but in a similar number of PCR-positive liver samples as the amount of MC-vector DNA was increased from 0.44 mg in pig A1 to 2 mg in pig C. For pig D1 the same conditions were applied as in pig C but with the 40% shorter vector MC.P3-luc; hence, an increased number of vector molecules were injected. Here we found 60% (45/75) of all liver samples to be positive 10 days after infusion. Again, there was a skewed distribution of positive samples with 100% positive specimens taken from the left liver lobes and 33% positive from the right and caudal lobes (pig D1 in Fig. 2). In pig D2, the same conditions were applied as in pig D1, resulting in 36/75 (48%) PCR-positive liver

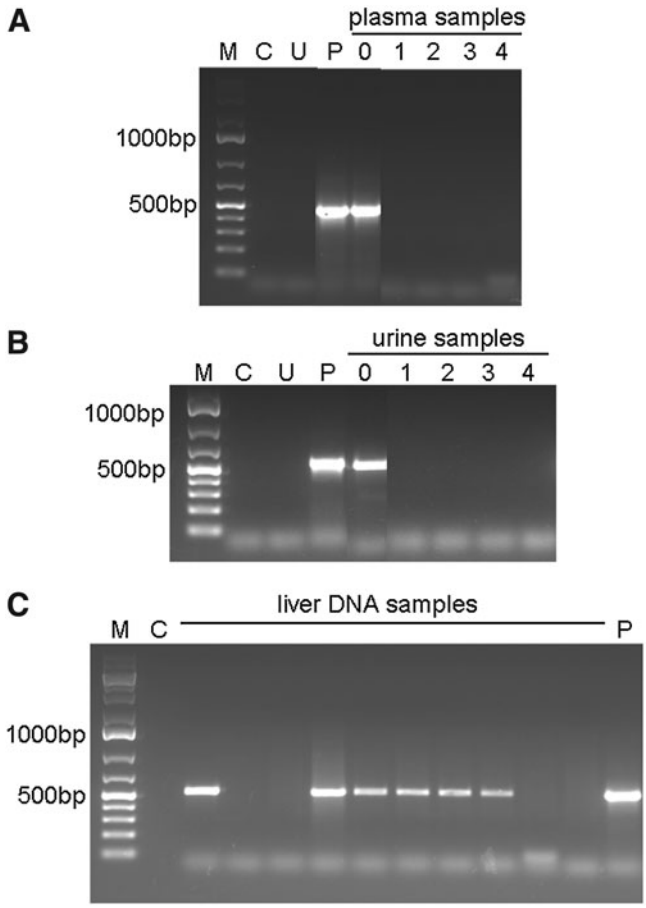

Figure 3. Detection of MC-DNA by end-point PCR results in plasma, urine, and liver upon MC-vector infusion. **(A)** Results of end-point PCR in plasma showed a positive signal right after injection (0) but was negative for plasma samples after 1, 2, 3, and 4 weeks (exemplified for pig B1). Water (C) and plasma from an untreated control pig (U) served as negative controls; MC.CMV-luc added to a plasma sample served as positive control (P). **(B)** PCR in urine gave the corresponding result to the plasma PCR, as it was positive immediately after injection (0) but no further positive results were found in urine samples after 1, 2, 3, and 4 weeks (exemplified for pig B2). Water (C) and untreated urine (U) served as negative controls; MC.CMV-luc added to a urine sample served as positive control (P). **(C)** PCR in liver DNA samples illustrating positive and negative results (exemplified for pig B1). MC.P3-luc in mouse liver served as positive control. **(A–C)** C, negative control (water); M, marker; P, positive control; U, untreated sample (plasma or urine) as additional negative control.

samples in this animal that had even a much larger liver at the time of euthanasia (400 g in pig D2 compared with 135 g in pig D1; Table 1). Although our observations are based on the limited number of animals we were allowed to use for these studies, we found stable liver transduction to be dependent on the flow rate, on the amount of vector injected, and/or on the MC vector size.

Analysis of MC-vector DNA in body fluids of MC-transduced pigs

When plasma was investigated, MC-vector DNA could be detected by PCR in samples obtained directly after vector infusion but not when blood was analyzed during postoperative monitoring or on the day of sacrifice (Fig. 3A). The same was observed in urine; that is, PCR-positive samples confirmed the presence of MC-DNA in the urine collected 10 min after MC-vector injection but samples were negative thereafter during postoperative monitoring period or upon sacrifice (Fig. 3B).

Impact of liver-targeted hydrodynamic gene delivery on liver toxicity and histology

We examined acute and long-term liver toxicity by enzyme marker activity and histological H&E staining, respectively. All CRP values after injection remained normal (<4 mg/liter). Furthermore, neither acute adverse effects such as changes in liver transaminases (AST and ALT) and LDH activities as measured before and during the entire postoperative monitoring (of 10 days or 4 weeks; not shown), nor any signs of necrosis in resected liver tissue were observed (Fig. 4). This confirmed that the infusion procedure left a healthy and intact liver in treated pigs, similarly as has been described by others[17,24] and to what we observed for the mouse liver upon hydrodynamic tail vein injection of MC-vectors.[16]

Luciferase activity and copy number per diploid genome in liver

A quantitative assessment and correlation of copy number per diploid genome with luciferase

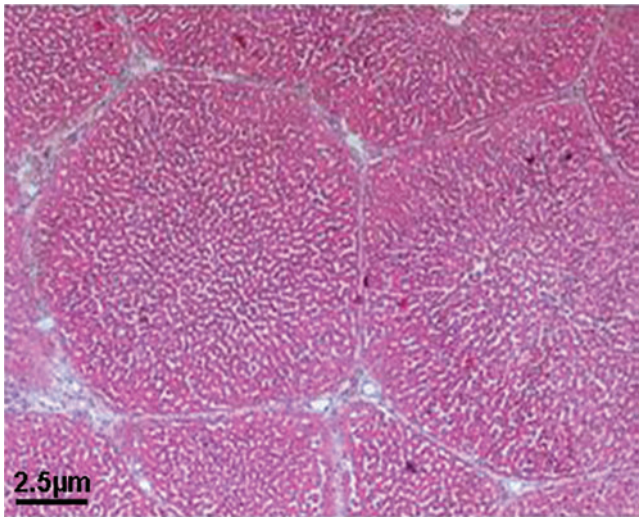

Figure 4. Liver histology. Hematoxylin–eosin-stained liver tissue sample obtained from pig B1 at the time of sacrifice at 28 days after vector DNA infusion. There are no signs of necrosis in the liver tissue and histology looks perfectly normal. The figure represents a 2.5-fold magnification. Color images available online at www.liebertpub.com/hgtb

activity in liver of pigs infused with the various MC vectors is summarized in Table 3A and B. With the exception of pig D1 (see below), all vector genomes per diploid liver genome were found to be 10^{-3} or lower, that is, undetectable in some liver samples. Luciferase activity in representative samples from each liver lobe in pigs A1, B1, and C, each transduced with a construct driving luciferase expression from the CMV promoter, was negative, which was expected as the CMV promoter does not sustain long-term expression and is eventually shut down in hepatocytes.[26] In contrast, vector MC.P3-luc, expressing luciferase from the strong liver-specific promoter P3,[22,16] resulted in low but stable transgene expression in pig D1 at least in both left lobes where we found the highest vector copy numbers. In detail, samples 37 and 42 within the left middle lobe showed 8.42 and 3.11 RLU/μg protein, respectively, and in the left lower lobe, 4 samples showed luciferase activity (samples 52, 54, 56, and 61 showed 1.52, 1.52, 6.25, and 1.06 RLU/μg protein, respectively). All 75 samples from pig D2, which received the same MC.P3-luc as pig D1, had no stable luciferase expression at 10 days after injection because of the low copy numbers that were comparable to pigs A1, B1, C, and D1.

As we observed stable and long-term expression of transgenes driven by the same liver-specific promoter P3 upon hydrodynamic tail vein injection of naked DNA/MC vectors in the mouse liver[16], we wanted to compare transfection efficiency between mouse and pig liver. Our best results were obtained with pig D1, in which 2 mg of MC.P3-luc was injected into a whole liver of 135 g (Table 1), which is equivalent to approximately 15 μg of vector DNA per gram of pig liver. We thus injected a proportionate amount of MC.P3-luc into a wild-type mouse, that is, 15 μg (with a liver of 1 g). Upon analysis of the whole mouse liver, we found about 10 copies per diploid hepatocyte genome in the mouse with a luciferase activity of 5692 RLU/μg protein. As we observed in pig D1 samples (no. 42 or 54 in Table 3B) each approximately 3 RLU/μg protein and one vector copy per diploid genome, that is, 10 times less genome copies per hepatocyte than in our mouse infused with the same vector, it can be estimated that MC.P3-luc expressed the luciferase $\sim$600-fold higher in murine than in pig hepatocytes. It remains to be determined though whether this is because of different promoter strength in the two species or because of other factors that might influence transgene expression.

DISCUSSION

Metabolic liver disorders are often life-threatening and curative therapies are scarce, rendering novel therapies urgently needed. Already for almost two decades, gene therapeutic approaches provoked much hope, but only recent advances in the field seem to justify the huge expectations.[27] While most gene therapy trials use viral vectors to transfect target cells, naked DNA/MCs were used as an alternative to reduce immunogenic complications of repeated viral vector applications or the risk of mutagenesis.[14,15] As MCs do not contain any bacterial or viral backbone, the safety of the nonviral vectors outmatches viral vectors used far more often for gene therapy until now. MCs can therefore be regarded as an alternative to "classical" viral vector-based gene therapy offering perspectives for many genetically determined (liver) disorders, which currently lack curative treatment.

However, since naked DNA lacks the natural capability of viral vectors to easily cross eukaryotic cell membranes, a different technical approach is required in order to achieve sufficient transfection of target cells.[28] One alternative to deliver naked DNA can be the application of a high-pressure injection. In the case of murine models, this was refined to the method of hydrodynamic tail vein injection, which was shown to successfully lead to a long-term cure of a liver metabolic disease, phenylketonuria (PKU), in a murine model.[16]

We performed this study to transfer the aforementioned successful method from mice to larger animals in order to finally be able to treat humans, or more specifically infants, rendering an alternative injection technique necessary since tail vein injections are not feasible in humans. To develop such method, we have chosen the pig model as ideally suited since these animals feature a similar anatomy, and have an immune system resembling humans more than mice.[29] As we intended to target the liver, and here specifically the periportal hepatocytes since several metabolic liver diseases are exclusively expressed here, the injection of naked DNA had thus to be transferred to a direct delivery into the portal vein. The technique of hydrodynamic injection into liver via portal or hepatic veins was already used successfully in rabbits, dogs, small pigs, larger (adult) pigs, and in humans (for a recent overview discussion, see ref.[19]). Here we successfully established, to our knowledge, for the first time the procedure of hydrodynamic portal vein injection in small pigs at the age of 4 weeks immediately after weaning.

The first challenge of hydrodynamic portal vein injection in small pigs directly after weaning was the size of the portal vein that required a thin catheter still allowing sufficient flow volumes. As this technique should eventually be established for use in human newborns or infants, establishing the method under similar conditions seemed crucial. Next to the establishment of a safe surgical catheter placement into the main portal vein, a sufficient hydrodynamic injection resulting in significant increases of the portal vein pressure had to be ensured in order to successfully transfer MCs into the periportal liver cells. As described by others,[17,18,30] this was only achieved after blockage of the vascular in- and outflow of the liver, a procedure that was tolerated well in our pigs. Thus, clamping of the portal vein distal to the inserted catheter, the hepatic artery, and the suprahepatic inferior vena cava was needed. Only if this rigorous clamping was applied, a sufficient increase of the pressure at the tip of the catheter was achieved allowing the assumption of increased intrahepatic pressure as also suggested by swelling of the entire organ. Alternative vein accesses were evaluated such as the splenic or gastroepiploic vein; however, they showed no permanent success rate, as depending on the angle of the junction of the splenic and the portal vein, it was often impossible to insert the catheter properly.

While single variables of the procedure were adapted during the study, the majority of the parameters were kept constant: in detail, (1) the age of the animals at the time of surgery has been kept constant as all were operated immediately after weaning; (2) the weight of the animals was as constant as possible in the given circumstances; (3) the injection volume (30 ml) was identical in all animals; (4) there were only two different conditions during injection, 20 ml/sec in 1.5 sec or 10 ml/sec in 3 sec; (5) there were only two different vector constructs used, either driven by a CMV promoter or by the liver-specific promoter P3; (6) there were only two time points for euthanasia (28 days or 10 days).

As demonstrated by PCR, an efficient transfection of liver cells was achieved by hydrodynamic injection. This effect was dose dependent, as we saw higher numbers of positive samples in animals injected with higher doses of MCs. In addition, the efficacy was dependent at least to some extent also on the flow rate of infusion, as injection of pigs D1 and D2, which were injected with 2 mg MC.P3-luc at a flow rate of 10 ml/sec, showed 60% and 48% PCR-positive liver samples, respectively. This confirms other reports of higher efficacy in experiments performed at lower flow rates.[30]

Interestingly, the distribution of positive samples was shifted to the left liver lobes. Since placement of the catheter within the main trunk of the portal vein was confirmed by angiography, we consider the skewed distribution as a result of the high-pressure injection directing more of the MC (and thus more DNA) to the left lobes of the liver according to the anatomical composition of the portal branches. An alternative explanation would be that higher injection volumes would be needed to achieve a more equal distribution.

Overall, the efficacy of our method needs to be improved as reflected by the number of PCR-negative samples. One reason for the limited efficacy may be the too low amount of DNA injected, as we found a dose-dependent transfection efficacy. Alternatively, it may require a more prolonged injection pressure than just between 1.5 and 3 sec, as applied in our settings. According to the experience made by others, our procedure of intraportal high-pressure injection may still be suboptimal with respect to the flow rate and volume, and the maximum pressure and time of the injection.

To investigate whether there was any washout of the injected MCs, we analyzed urine and blood samples. To avoid loss of single MCs, we established a method to subject urine and plasma directly as PCR template instead of the commonly used DNA isolation protocols. We could confirm reliable PCR amplification as long as the concentrations of PCR-inhibiting substances in the specific body fluids are considered and samples diluted accordingly. Since we did not detect MC-DNA in any of the urine and plasma samples, the positive results obtained in liver tissue reflect MC survival within hepatocytes as degradation occurs, as expected from previous studies, outside cells. Importantly, all other organs apart from liver were negative.

In addition to the presence of MC-DNA after 10 days and 4 weeks in all pigs investigated, we detected a low but stable luciferase activity in samples from only pig D1. Consistent with this low luciferase expression, quantitative PCR showed appropriate copy numbers per diploid genome in liver samples from pig D1. The low luciferase expression underlines our interpretation that the main future aim for the hydrodynamic approach must be a further improvement of the methods' efficacy. This is in particular advisable as experiments in mice show a much better transduction efficacy as illustrated by the higher copy numbers and higher levels of lucif-

erase activity. In this context, one should emphasize that the injection procedures were different in pigs and in mice. This difference includes the injection approach (portal vein in pigs and tail vein in mice), the volume used for injections in comparison to body weight (0.6% in pigs and 10% in mice), and the unknown maximum pressure in mouse liver under hydrodynamic tail vein injection. All these different parameters make it difficult to compare results in pigs with mice data.

Interestingly, pig D1 (and also pig D2) was transduced with an MC vector expressing the luciferase from a strong liver-specific promoter, which was not the case for other pigs that were transfected with the non-liver-specific CMV promoter. In mice, we observed shutdown of luciferase expression from the CMV promoter in the same MC vector in less than 10 days after hydrodynamic tail vein injection (unpublished observation). Although we have at this time no further data on the performance in pig liver, we speculate that a CMV promoter shutdown is likely to occur and might explain the absence of luciferase expression in those livers. Further experiments with liver-specific promoters and other optimized constructs will eventually show whether any transfected activity will finally achieve therapeutic levels. As an alternative to hydrodynamic injections, normodynamic interventions applying encapsulation of MCs can be considered.[31]

In conclusion, high-pressure portal vein injection of MC DNA is a safe procedure in small pigs immediately after weaning. Establishing this method in pigs with a weight comparable to human newborns should encourage re-evaluation of this method also for human patients. The efficacy of the method, however, needs to be increased in order to finally use this method for therapeutic purposes. If the intraportal injection after reducing the invasiveness and improving the efficacy still is safe, it might become an auspicious therapy for several metabolic liver disorders.

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AUTHOR DISCLOSURE

The authors declare that there is no conflict of interest. No competing financial interests exist.

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